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<b>(21) International Application Number:</b> PCT/DK91/00382 <b>(22) International Filing Date:</b> 5 December 1991 (05.12.91)  <b>(30) Priority data:</b> 90610072.2 5 December 1990 (05.12.90) EP <i>(34) Countries for which the regional or international application was filed:</i> AT et al.  <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only) :</b> LØVBORG, Uffe [DK/DK]; Grantofteparken 60, DK-2750 Ballerup (DK).		<b>(74) Agent:</b> NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsværd (DK).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), BG, BR, CA, CH (European patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LU (European patent), MC (European patent), NL (European patent), NO, PL, RO, SE (European patent), SU*, US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PROTEINS WITH CHANGED EPITOPES AND METHODS FOR THE PRODUCTION THEREOF  <b>(57) Abstract</b>  <p>The invention provides methods for selecting where in the amino acid sequence of a protein to modify in order to obtain protein variants evoking a reduced immunological response, and protein variants subsequently produced. Also compositions containing the variants and their use in especially detergents and in medicine is disclosed.</p>		

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**Title: PROTEINS WITH CHANGED EPITOPES AND METHODS FOR THE  
PRODUCTION THEREOF**

5

**FIELD OF THE INVENTION**

This invention relates to methods for modifying proteins, especially enzymes that are used industrially, and proteins  
10 used in medicine, and the modified proteins produced thereby, compositions containing such protein variants, and the use of the variants in various fields, including medicine. According to the invention the proteins are epitope mapped using immunological and proteochemical methods and eventually their  
15 amino acid sequence is changed through genetic engineering thereby changing their immunological activity in order to make them less immunogenic and thereby reduce the risk of provoking allergic responses in animals, including man, subjected to exposure to the enzymes of the present inven-  
20 tion.

**BACKGROUND OF THE INVENTION**

25 Various proteins, such as enzymes are used increasingly in industry and householding. Being proteins they will be able to stimulate an immunological response in man and animals.

Other proteins, such as hormones are used increasingly in  
30 medicine for the treatment and/or diagnosis of various conditions of illness and disease, whereby these proteins are injected into or otherwise presented to the immune system of animals, including man.

35 Depending on the way of presentation the stimulation can lead to production of various types of antibodies, and to a cellular response too. Of these routes at least one, being the

one type of antibody can have adverse effect in man and animals. The production of IgE and maybe IgG4 can lead to an allergic state, giving symptoms like f.ex. rhinitis, conjunctivitis or other.

5

It cannot be excluded that other immunologically based adverse reactions will be seen with the increased use of these proteins.

10 These drawbacks in the use of proteins have been known for many years and various solutions have been used for solving these.

Within the field of industrial enzymes the most frequently  
15 used method for avoiding problems with allergic reactions from exposure to the products has been confectioning the enzymes in various ways by immobilizing, granulating and coating the enzymes thereby avoiding any release of the proteinaceous material during normal handling and storage.

20

However, this solution poses various problems in relation to bringing the enzyme into contact with the material with which it is meant to interact, such as bringing the enzyme into solution etc., and also some release of the enzyme may occur  
25 provoking an allergic reaction in subjects sensitive to an exposure.

Within the field of medicine a much used method has been to use proteins of especially human or corresponding animal  
30 origin or at least of the same primary structure as the human (or the animal in question) protein.

This has proven to be successful in many instances, but it is not always possible to establish the existence of an animal  
35 equivalent to the protein in question, or it has been found that certain modified proteins possess certain advantages over the native protein. In such instances the risk of provo-

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king an allergic response in the subject receiving treatment or being diagnosed exists.

Consequently a need exists for developing proteins that  
 5 provoke less or no allergic reactions, while still retaining their original activity to a degree where they still are functional and may be used according to their original intent.

10 Those parts of a protein molecule that are recognized and bound immunologically are called epitopes. For molecules in the range of f.ex. 30000 Daltons there might be as many 12 epitopes.

15 Epitopes are being bound by immunological cells and by antibodies. Some epitopes are more important than other, these are called major in contrast to minor epitopes.

It has been found that slight changes in the epitopes will  
 20 affect the binding strength in these bindings (Walsh, B.J. and Howden, M.E.H. (1989): A method for the detection of IgE binding sequences of allergens based on a modification of epitope mapping, Journal of Immunological Methods, 121, 275-280; Geysen, H.M., Tainen, J.A., Rodda, S.J., Mason, T.J.,  
 25 Alexander, H., Getzoff, E.D. and Lerner, R.A. (1987): Chemistry of Antibody Binding to a Protein. Science. 135, 1184-90; Geysen, H.M., Mason, T.J. and Rodda, S.J. (1988): Cognitive Features of Continuous Antigenic Determinants. Journal of molecular recognition. 1, 32-41.

30

This may result in a reduced importance of such a changed epitope, maybe converting it from a major to a minor epitope, or the binding strength may even be decreased to the level of high reversibility, i.e. no efficient binding. This pheno-  
 35 menon may be called epitope loss.

The above investigations were all performed on synthesized

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peptides mimicking the epitopes in question and variants thereof in order to establish the relative importance of the amino acid residues in the epitope being investigated, and consequently these investigations do not prove any effects to the epitopes in their native environment as parts of the complete protein, where phenomena only found in the tertiary structure of the protein, such as folding or the establishment of salt bridges etc., are in function.

10

#### SUMMARY OF THE INVENTION

The object of the invention is to provide for methods for selecting where in the amino acid sequence of a protein to modify in order to obtain protein variants evoking a reduced immunological response, and these protein variants.

The present invention consequently in a first aspect relates to a method of producing protein variants evoking a reduced immunogenic response in animals including man in comparison to the response evoked by its parent protein.

For this the protein is epitope mapped using immunological and proteochemical methods, and the various epitopes are determined and characterized.

Subsequent to this at least one of said epitopes is changed through mutation of a DNA molecule coding for the expression of said parent protein, or through synthesis of a DNA molecule coding for the expression of said variant protein. this is performed by using well established techniques known in the art of protein engineering.

The mutated or constructed DNA molecule is subsequently inserted into a vector for transformation of transfection into a suitable host, wherein said vector is functional or

whereby said mutated or constructed DNA molecule is integrated functionally into the genome of said host, and the protein variant of interest is expressed in the host.

5 Finally the protein variant is recovered and purified.

In a second aspect the invention relates to the proteins produced by the above method. Under this aspect industrial enzymes, such as detergent enzymes, e.g. proteases, lipases, 10 cellulases, amylases, or oxidases, process enzymes, e.g. amylases, lyases, lipases, or cellulases, medicinal proteins, e.g. hormones, e.g. insulin, HCG, or growth hormone, or medicinal enzymes, e.g. factor V, factor VII, factor VIII, or other proteins, e.g. interleukins, or interferons, are of 15 special interest.

In a third aspect the invention relates to compositions comprising the proteins of the second aspect, such as detergent compositions, or compositions for use in preventive 20 and/or alleviating therapy and/or diagnosis of various conditions in the animal body, including man.

In a fourth aspect the invention relates to the use of such compositions in preventive and/or alleviating therapy and/or 25 diagnosis of various conditions in the animal body, including man.

### 30 BRIEF DESCRIPTION OF THE DRAWING

The invention will be explained and illustrated in further detail in the following parts of the specification including the specific examples and the appended drawing, wherein

35

Figures 1 to 6 show plots of the binding of a number of enzyme variants to a reference antiserum as a function of

their concentration

DETAILED DESCRIPTION OF THE INVENTION

5

According to the first aspect of the invention epitope mapping is used to locate and characterize the various epitopes functionally present in a protein. Thereafter this information is used for selecting which amino acid residues in the  
10 epitopes should be changed.

When the changes have been implemented through the now well established techniques of genetic engineering, and the protein variants have been produced, immunological and proteo-  
15 chemical techniques are used to analyze the new protein variants and determine whether the changes have led to switches from major to minor epitopicity or even to epitope loss.

This information is again used to decide whether the protein  
20 variant(s) produced correspond to demands established for the protein, or, whether more or other changes have to be implemented.

Through the invention it has thus been made possible to  
25 produce proteins, especially industrial enzymes and medicinal proteins that will present a reduced immunologic, such as allergenic, potential risk to the environment and animals subjected to exposure to the protein(s) in question.

30 The protein or enzyme variants of the invention will therefore present lower risk to man (and animals) be it in the production, usage or to the environment.

35 In performing protein mapping the protein of interest (called the reference protein) and variants thereof, made by genetic engineering or by chemical modification, are used for the



production of antibodies. Antibodies can be polyclonal (like antisera) recognizing many epitopes in an antigen and cross reacting with other often related antigens, monospecific recognizing a single antigen, epitope monospecific recognizing a single epitope, or monoclonal recognizing a single epitope and produced through fusion of cells producing the antibody and immortal cells, such as carcinoma cells.

Polyclonal antibodies will react to the protein antigen in a polyspecific manner, i.e. there will be many specificities each reacting with each own epitope in the antigen or showing different reactivities to different related epitopes. Also, polyclonal antibodies will often cross-react with related antigens. Monospecific antibodies are polyclonal antibodies isolated according to their specificity for a certain antigen, such monospecific antibodies will normally only be specific to a very limited number of epitopes, and often only specific to one epitope.

Epitope mono specific antibodies are polyclonal antibodies isolated according to their specificity for a certain epitope. Such epitope mono specific antibodies will only be specific to one epitope, but they will often be produced by a number of antibody producing cells, and are consequently not identical.

Monoclonal antibodies are epitope specific antibodies produced by the now well established technique of cell fusion between an antibody producing cell and an immortal cell. All monoclonal antibodies produced by one clone are identical.

The antibodies produced can bind the immunizing protein antigen. Furthermore, if fully or partially identical epitopes exist in the other proteins, the antibody will be able to bind to these too. If there is complete identity the recognition and binding will be identical. If there is partly identical epitopes the recognition will be different and the

binding strength will be lower. If the epitope is not present the antibody will not bind.

The mapping using polyclonal antibodies, can be divided into two phases:

- i) Measure the reactivity of the antibody preparations toward all proteins of interest.
- ii) Measure the reactivity left over to react with one antigen after reaction with another.

10

The results from (i) will provide information about the immunogenic and allergenic potential of the variants investigated. According to this some variants exhibiting a reduced potential could prove to be interesting protein variants, whereas others exhibiting an increased potential are deemed not to be interesting from an immunological viewpoint.

- The results from (ii) will provide information concerning:
- iia) changes in epitope(s) showing which epitopes are more or less immunogenic/allergenic.
  - iib) loss of epitope(s) (even the highest concentration of one antigen will not eradicate all reactivity to reference antigen), or
  - iic) establishment of new epitope(s) (even the highest concentration of reference antigen will not eradicate all reactivity to a variant).

From this information it can be decided which variants can be used for the production

30

The selected protein variants may be produced by methods which by now are well known to the person skilled in the art of protein engineering, and described in numerous publications, such as International Publication No. WO/06279 (NOVO INDUSTRI A/S), and International Patent Application No. PCT/DK90/00164 (NOVO-NORDISK A/S) for both of which relevant sections are hereby incorporated in their entirety by refe-

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rence.

### EXAMPLES

5

The reference protein antigen chosen was SP436, a variant of the alkaline protease, subtilisin 309, whose construction and production is described in detail in the above mentioned International Publication No. WO/06279 (NOVO INDUSTRI A/S), where it was designated (i). The SP436 variant comprises in respect of the wild type subtilisin 309 two changes in the amino acid sequence, i.e. G195E+M222A. International Patent Application No. PCT/DK90/00164 (NOVO-NORDISK A/S) shows the production of further variants made by genetic engineering. The wild type enzyme is produced by normal fermentation, and the antibodies are polyclonal from rat.

The SP436 molecule is a protein comprising 269 amino acid residues, and it has in comparison to the well known subtilisin BPN' 6 deletions. For further reference to the amino acid sequence of various subtilisin reference is again made to International Publication No. WO/06279 (NOVO INDUSTRI A/S), and International Patent Application No. PCT/DK90/00164 (NOVO-NORDISK A/S), wherein the amino acid sequences for a number of proteases, a numbering system for subtilisin enzymes based upon the sequence of the subtilisin BPN', and a notation for indicating changes in the amino acid sequences are indicated. The numbering and notation therefrom will be followed throughout this specification and appended claims.

30

### IMMUNIZATIONS.

Rats were selected as test animal due to the fact that according to the literature these are the only normal laboratory animal that are capable of binding human IgE onto its mast and basophile cell membranes, and at the same time having IgE

that will bind to human mast and basophile membranes.

The animals were divided into 12 groups each of 3 rats. For the immunizations the wild type (wt) subtilisin 309 and 11 variants thereof were selected. These are indicated in TABLE I below:

TABLE I

Subtilisin 309 variants used for immunization

10	Grp No.	"Variant"	Adjuvant	Change in respect of wt
	1	SP436	Freund	G195E+M222A p - u u
15	2	S001	-	G195E p -
	3	S003	-	R170Y + p
	4	S005	-	K251E + -
20	5	S015	-	K235L + u
	6	S026	-	E136R - +
25	7	S033	-	E271Q - p
	8	S006	-	H120D + -
	9	S020	-	H120D+R170Y+G195E+K235L+K251E + - + p p - + u + -
30	10	S023	-	*36D+H120D+R170Y+G195E+K235L m - + - + p p - + u
	11	S028	-	D181N - p
35	12	WT	-	

-: negatively charged

+: positively charged

p: polar

40 u: unpolar

m: missing(deletion)

The injected quantity was invariably 30 µg/animal/immunization. Each animal received 6 injections.

45

All 12 selected proteins were injected once in Freund's Complete Adjuvant, once in Freund's Incomplete Adjuvant and four times in NaCl 0.9%.

- 5 Blood was harvested one week after each immunization except for the final exsanguination, which followed 5 days after the last immunization.

After clotting, the sera from all three animals in each group  
10 were pooled .

The analytical work described in the present report was on the

12 sera pools after the third blood harvest.

15

#### ANALYSIS.

The analytical work was performed in two series of analysis, A and D, both of which are ELISA techniques.

20

#### Series A:

One protein is used for coating the wells of one or more ELISA-plates. This protein can be the native (wildtype) or a  
25 variant.

The 12 different sera pools in this analysis are incubated in the coated wells. The sera have all been raised against different proteins. If the variants are similar the sera are  
30 expected to be similar in their reactivity pattern too. Each sera pool is tested in a dilution series in its own series of wells.

The potential binding of rat antibodies is visualized through  
35 binding of peroxidase labelled anti-rat antibodies.

If rat antibodies were bound to the enzyme coating, they will

be bound in proportional manner by the peroxidase labelled anti-rat antibodies.

The presence of colour in this way gives a proportional visual and measurable indication of presence of enzyme specific rat antibodies.

In a short step by step sequence the setup is:

- 1) Enzyme coating of solid phase.
- 2) Albumin blocking of residual binding spots on solid phase.
- 3) Incubating sera in dilution series, enzyme active anti-bodies being bound to the coated enzyme.
- 4) Peroxidase labelled anti rat(IgX) antibodies.
- 5) Development of colour.
- 6) Determination.

The 12 sera groups were tested for reactivity towards one kind of protein (i.e. wt or variant) according to the above set-up. One by one different proteins were tested with the 12 sera groups.

The response was compared to the sera group originally immunized with the given protein, i.e. the reference.

The results give information on antibody recognizability of the individual proteins. Division can be made into three kinds of reactivity relative to the reference, i.e. same/higher/lower reactivity. See TABLE II below. Because of the assay design the phenomena of epitope loss and/or epitope change (to give a decrease in binding strength) are indistinguishable from each other.

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TABLE II  
IgG  
Sera group no.:  
Variant: 1 2 3 4 5 6 7 8 9 10 11 12  
Selected dilution for all: 1280x  
SERIES A: AN OVERVIEW OF RESULTS

	1	2	3	4	5	6	7	8	9	10	11	12	REF.	GRP NO:
5														
WT	850	1152	1745	2486	2638	429	2361	2020	1186	1500	2004	977	12	
SP436	276	867	1933	2279	2832	287	1956	2526	1926	2491	2547	134	1	
S003	1265	1505	2614	2449	2079	218	1613	3178	1890	2146	2442	62	3	
S005	1600	1600	2097	2100	2580	237	1900	3435	2699	3075	2066	89	4	
10 S023*	2034	1295	2696	2700	2736	332	1469	2326	1754	2968	1716	115	10	
S001	1119	1204	1652	1941	2502	285	1762	2836	2359	1701	2262	45	2	
S026	888	944	1544	1721	2415	615	1556	2785	1332	1147	1646	40	6	
SP458	1111	930	1287	1600	2213	193	2129	2596	1278	1323	2052	33	none	
IgE														
15 WT	1017	1091	1216	1355	1396	460	1181	1786	1209	1264	1473	577	12	
SP436	341	680	1066	1143	1251	291	858	1185	1025	1350	1355	92	1	
S003	1135	1398	1452	1561	1726	646	1283	1693	1352	1499	1532	141	3	
S005	1006	1003	1316	1654	1672	412	1182	1909	1409	1587	1188	105	4	
S023	1117	1247	1500	1484	1241	333	940	1317	1460	1441	1398	56	10	
20 S001	836	1059	1298	1228	1490	324	1043	1509	1174	1172	1309	124	2	
S026	780	1033	1325	1374	1585	568	1205	1402	827	996	1188	103	6	
SP458	774	850	1092	1145	1517	388	1281	1289	790	1000	1205	83	none	

\* S023: sera 1 through 7 is multiplied with 1.5 to compare sera 8 through 12.

The IgG response (TABLE II) shows three effects :

- 1) Each sera group (except anti-SP436) reacts stronger with its own immunogen than with any of the other. Especially sera no 12 (anti-wt) show dramatic lower response to other proteins.
- 2) Some sera give in general higher responses than other.
- 10 This last feature can become very important together with the IgG and IgE distribution. Anyhow, it cannot be excluded to belong to some individuality in the responding animals. Such a feature is often expressed when only few animal sera are pooled (like in this case, three).
- 15 3) There is a heteroclitic effect for each of the tested proteins except S023. This means that sera from animals immunized with a protein that is not the test protein, will react stronger than the sera coming from animals immunized with the test protein (horizontal values).
- 20

This is a characteristic feature also seen in work with small synthesized peptides that are used to produce antibodies to native (larger) protein. Here it is explained by differences in conformation being in favour of the native molecule.

The IgE response (TABLE II) show effects comparable to (1), (2), and (3) mentioned for the IgG response.

- 30 Switch from one immunizing protein to another similar protein will for all except SP436 give lower IgG and IgE response. Switch from SP436 to another will increase this very signal, but only to a level comparable to the ones otherwise seen.
- 35 Furthermore there is a heteroclitic effect, which will be further discussed in connection with the following series D.



# SERIES A :

Selected seras were tested with one and the same variant in each analysis. The variants were used for solid phase coating  
5 at a concentration of 50 µg/ml (phosphate buffer), this gave a near-monolayer immobilization. Residual binding spots on the surface were blocked by bovine serum albumin (=BSA).

Sera were tested in dilution series, first dilution 20 to 800  
10 times, depending on sera strenght, and from this dilution in two-fold series. Phosphate buffer including blocking agent BSA and detergent.

Tracing performed by bound antivariant-antibody by mouse-  
15 anti-rat antibodies that are conjugated to peroxidase. (Kem-En-Tec cat. no. Y 3300 diluted 1000x in same buffer as used for seras).

Visualization was obtained through enzymatic reaction of  
20 peroxidase on OPD-substrate that is turning colored proportionally to peroxidase present, which is proportional to rat anti variant antibodies present.

Sera having high potential for reaction will give higher  
25 response than others, and this will make estimation of strenght and mutual reactivity possible.

## 4.A.4. SERIES A, analysis 2+3

30 Analysis was performed as decribed under methods. Calculation of dilutions giving equal response, and "normalizing" these to the reference (i.e. the reaction of the individual sera with its immunising variant).

35 A low figure means the sera cannot be diluted as much as the reference, and a high figure that the sera can be diluted more than the reference. 49 therefore means that the serum

can be diluted only 0.49 times the reference reaction, e.g.  
490x for the sample in comparison to the reference 1000x.

Results from these experiments are indicated in TABLE III  
5 below:

TABLE III

		SERIES A (analysis 2 + 3)			DATA EXTRACTION						
10		AMINO ACID			FORWARD		REVER- SE		RE- SPONSE		
		EXCHANGE :			EXCHAN- GE:		EXCHANGE :		TYPE :		
	G195E				53			53			A
	R170Y				84			65			A
15	D181N				164			24			B
	K235L				114			56			B
	E136R				80			53			B
	E271Q				96			48			B
20											
	H120D				100			36			B
	E251K				59			91			C

	G195E+M222A			67			49			A
	E195G+R170Y			126			77			B
	E195G+E136R			106			50			B
5										
	Y170R+E136R			75			79			A
	E251K+H120D			91			90			A
10	E251K+D181N			134			74			B
	E251K+E271Q			118			59			B
15	Q271E+H120D			73			137			C
	D120H+D181N			137			71			B
20	D120H+E271Q			137			73			B
	N181D+K235L			79			128			C
25	E195G+A222M+R170Y			70			65			A
	E195G+A222M+E136R			70			59			A

5	H120D+G195E+K235L+K251E		89			95			A
	H120D+R170Y+K235L+K251E		109			61			B
	*36D+H120D+R170Y+G195E		56			103			C
10	*36D+R170Y+G195E+K235L		40			82			C
	H120D+R170Y+G195E+K235L+K251E		90			45			B
	*36D+H120D+R170Y+G195E+K235L		73			19			B
15	H120D+R170Y+A222M+K235L+K251E		72			82			A
	*36D+H120D+R170Y+G195E+K235L, E251K		76			82			A
	R136E+H120D+R170Y+G195E+K235L+K251E		75			73			A
20	Q271E+*36D+H120D+R170Y+G195E+K235L		50			95			B
	D36*+D120H+Y170R+E195G+L235K+D181N		117			41			B
25									

	forward exchange : amino acid exchange as listed to the left.										
	reverse exchange : amino acid exchange opposite to the listed.										
5											
	type :	A = exchanges gives nearly equal effect i both directions.									
		B = the reverse exchange is more important									
		C = the forward exchange is more important									
10											
	for all : if different from 100 this amino acid position is included in an epitope.										
		if <100 the epitope change means need for more antibody to give a response equal to the reference reaction.									
15											
		if >100 the epitope change means that there is a heteroclitic effect (affinity increase to this epitope).									

The interpretation of these results are discussed in detail  
20 below in the concluding remarks.

Series D:

Again one protein was used for coating the wells in ELISA  
25 plates. Furthermore only one sera pool was tested as the

reference. This serum was tested in only one dilution.

Before the sera were added to the coated wells, they were preincubated with the wild type or variant of the protein in another set of wells.

In these other wells dilution series of wild type or variants were incubated with the same concentration of the reference. Each well therefore contained serum plus either wild type or variant in a specific dilution from a dilution series.

The protein concentrations, were selected to assure a surplus of antigen in one end of the dilution series, and nearly no reaction in the other end. Consequently the protein can in some wells block all rat anti-protein reactivity, if it corresponds in specificity. In other wells more and more rat antibody will remain unreacted. If the co-incubated protein is very different from the one used to produce the rat antibodies, unreacted antibodies will remain in all wells, independent of the concentration used.

After the co-incubation of sera plus diluted proteins, the reacted mixture was transferred to the coated wells.

If rat antibody activity is left over it will bind to the coated protein in the wells, and eventually the rat antibodies are bound by peroxidase labelled anti rat(IgX) antibodies; and development performed as above in Series A.

The results in this assay indicate whether the co-incubated protein is related to the coated or not. Both with respect to epitope identity (partly or fully) or epitope presence.

The variants used in this series are indicated below in TABLE IV:

TABLE IV

Subtilisin 309 variants used in Series D

Changes in amino acid sequence compared to WT:

WT		
5	SP436	G195E+M222A
	SP458	M222A
	S001)	G195E
	S003)	R170Y
	S005)	K251E
10	S006)	H120D
	S012)	R170Y+G195E+K251E
	S015)	K235L
	S019)	H120D+R170Y+G195E+K235L
	S020)	H120D+R170Y+G195E+K235L+K251E
15	S021)	*36D
	S022)	*36D+R170Y+G195E+K251E
	S023)	*36D+H120D+R170Y+G195E+K235L
	S024)	*36D+H120D+R170Y+G195E+K235L+K251E
	S025)	*36D+H120D+G195E+K235L
20	S026)	E136R
	S027)	E89S
	S028)	D181N
	S033)	E271Q
	S046)	Y209L
25		

One sera group was used per assay, consisting of 22 subassays. In each sub-assay the sera were absorbed in liquid phase with wt or one of the variants in a dilution series. Finally the remaining antibodies were tested towards one and the same protein all over.

Each sub-assay therefore results in removing antibody reactivity and estimating what is left over. In this set up one sera group is being absorbed with all 21 proteins, and finally tested with the same type originally used for immunization.

Loss of epitopes in the absorbed protein compared to the tested protein will mean positive results even with the highest concentration of absorbing protein. As indicated in Figs. 1 to 6 the plots will for such variants level off not reaching the full effect seen in the reference absolute absorbtion.

Change of epitopes can mean lowering of binding strength. Therefore the sub-assay plots will be positioned different on the concentration axis, giving a titer difference in comparison with the reference absolute absorbtion.

So far only antisera to SP436 have been tested.

- 15 In Figures 1 through 6 the effect of absorbtion is plotted. The first well is without any variant or wt to absorb, i.e. an internal control. The following wells contain increasing concentrations of absorbing protein.
- 20 There is basically two types of plots. One is with decreasing values all over. Another is levelling off, leaving some response even in the presence of the highest concentration of absorbing protein. The first type will correspond to change of epitopes, whereas the second type will correspond to loss of epitope (meaning a general lowering of binding energy, enabling a high degree of reversibility in antibody binding).

From Figures 1 through 6 it is obvious that the following S numbers "level off" : S003, S012, S019, S020, S022, S023, S024 and S026. S026 contains the variant E136R and is the only variant in that position tested, therefore S026 is not included fully in the evaluation.

In TABLE V the effect of absorbtion is listed for all 21 proteins including the reference SP436 sorted in three parts.

The first part gives the effect of an amino acid change in



position nos. 195 and 222, and combinations with other single position changes.

The second part gives change of position no. 170 in combination with all other tested changes.

The third part gives change of position no. 251 in combination with all other tested changes.

10 The test serum was serum group no 1 (anti-SP436).

Results are measured as titer at one fixed read-out value, and recalculated to the absorbing capacity relative to the reference (in percent).

TABLE V

SERIES D, coat: SP436, sera group no. 1

All values are relative to sera group no 1 (= ref.).

All values are absorbance capacity relative to the reference.

5	Variant	IgG	IgE	Amino acid exch.
		v.40NSVU	v.40NSVU	rel. to SP 436
	SP458	70	59	195
	S001	70	87	222
10	WT	9	10	195, 222
	S021	27	31	36, 195, 222
	S027	104	380	89, 195, 222
	S006	26	30	120, 195, 222
	S026	0	0	136, 195, 222
15	S003	26	40	170, 195, 222
	S028	102	220	181, 195, 222
	S046	120	150	195, 209, 222
	S015	30	36	195, 222, 235
	S005	74	67	195, 222, 251
20	S033	74	29	195, 222, 271
	S003	26	40	170, 195, 222
	S012	14	20	170, 222, 251
	S019	23	125	120, 170, 222, 235
25	S020	20	88	120, 170, 222, 235, 251
	S022	4	11	36, 170, 222, 251
	S023	4	4	36, 120, 170, 222, 235
	S024	2	6	36, 120, 170, 222, 235,
	251			
30				
	S005	74	67	195, 222, 251
	S012	14	20	170, 222, 251
	S020	20	88	120, 170, 222, 235, 251
	S022	4	11	36, 170, 222, 251
35	S024	2	6	36, 120, 170, 222, 235,
	251			

SUBSTITUTE SHEET

## RESULTS OF SERIES D.

Taking amino acid change one by one from TABLE V, absorption capacity being compared to the reference :

5 (ND = not distinguishable in present set-up)

No. 36:

	with 195+222 (S021)	ND
	with 170+222+251 (S022)	ND
10	with 120+170+222+235 (S023)	absorb less IgE
	with 120+170+222+235+251 (S024)	absorb less IgE

No. 89:

	with 195+222 (S027)	absorb more IgG , and absorb much more IgE
--	---------------------	---

15 No. 120:

	with 195+222 (S006)	ND
--	---------------------	----

no. 170:

	with 195+222 (S003)	ND
--	---------------------	----

no. 181:

20	with 195+222 (S028)	absorb more IgG, and absorb much more IgE
----	---------------------	--

No. 195:

	alone (SP 458)	absorb little less IgG, and absorb little less IgE
25	with 222 (SP 436)	absorb much less IgG, and absorb much less IgE

No. 222:

30	alone (S001)	absorb little less IgG, and absorb little less IgE
----	--------------	--

No. 235:

	with 195+222 (S015)	ND
--	---------------------	----

35 No. 251:

	with 195+222 (S005)	absorb little more IgG, and absorb little more IgE
--	---------------------	--

	with 36+120+170+222+235 (S024)	ND
--	--------------------------------	----

40 no. 271:

	with 195+222 (S033)	absorb more IgG
--	---------------------	-----------------

The results must be compared with data in TABLE VI, where the inter-atomic distances between  $C_\alpha$ 's are listed.

The epitope size is typically 10-15 Å in radius, and the 5 amino acids are exposed like in a field.

Combining this information with a 3-dimensional (3D) view, it will be possible to estimate which amino acids belong to the same epitope, and therefore will be bound by the same antibody.

TABLE VI  
INTERATOMIC DISTANCES BETWEEN C<sub>α</sub>'s in Å

AA no.	36	89	120	136	170	181	195	209	222	235	251	271
36	0	13,6	18,2	23,5	27,3	26	30,6	8,5	15,6	24,3	29,7	28,6
5 89	13,6	0	9,9	25,9	26,8	27,6	28,4	18,3	18,5	12,6	23,3	20,7
120	18,2	9,9	0	18,4	20,3	27,8	21,6	24,4	19,5	8,5	17,6	20,2
136	23,5	25,9	18,4	0	9,4	29,8	14,3	30,6	22,7	24,5	22,1	32
170	27,3	26,8	20,3	9,4	0	22	5,9	28,6	17,8	23,7	16,4	27,2
181	26	27,6	27,8	29,8	22	0	20,5	20,9	11,1	26,5	18,9	19,3
10 195	30,6	28,4	21,6	14,3	5,9	20,5	0	31	18,9	23	12,5	24,4
209	8,5	18,3	24,4	30,6	28,6	20,9	31	0	12,8	27	29,7	27,2
222	15,6	18,5	19,5	22,7	17,8	11,1	18,9	12,8	0	20,9	18,4	19,6
235	24,3	12,6	8,5	24,5	23,7	26,5	23	27	20,9	0	14,2	13,4
251	29,7	23,3	17,6	22,1	16,4	18,9	12,5	29,7	18,4	14,2	0	12,8
15 271	28,6	20,7	20,2	32	27,2	19,3	24,4	27,2	19,6	13,4	12,8	0

Distances to/from no 36 are estimated as mean of (35 + 37) as the subtilisin 309 database does not include 3D coordinates of no 36 (not present in wt).

Initially nos 120 + 235 seem to cooperate in one epitope, and  
nos 195 + 251 in another epitope.

Furthermore nos 89 and 181 both will give much higher absorb-  
5 tion of both IgE and IgG. No 251 little more of both, and no  
271 little more of IgG.

Amino acid no 170 is changed in all the other cited nos., -  
leading to loss of epitope. Even the highest concentration of  
10 these proteins will not remove all antibodies from the prepa-  
ration.

This single epitope accounts for approximately 30 % of the  
reactivity, therefore it can be expected that the total  
15 number of epitopes is low.

Also, it seems as if position 136 is connected with a major  
epitope (cf Fig. 4). Since S026 is the only variant wherein  
position 136 is changed, a definite conclusion must await  
20 further study.

In TABLE VII below the probability for pairs of positions  
investigated here belonging to the same epitope is indicated

TABLE VII

PROBABILITY FOR BEING IN THE SAME EPITOPE ( <9.9 Å: high, 10-15 Å: medium)												
AA no.	36	89	120	136	170	181	195	209	222	235	251	271
36		medium						high				
5 89			high							medium		
120										high		
136					high		medium					
170							high					
181												
10 195									medium		medium	
209										medium		
222									medium			
235											medium	med
251												med
15 271												

From the above the following amino acid residues are selected for being changed in order to influence the immunological potential of subtilisin 309.

5 non-polar: 129, 131, 151, 152, 162, 168, 169, 172, 174,  
175, 176, 194, 196,  
polar: 127, 128, 130, 153, 154, 161, 163, 167, 171,  
173, 193, 195,  
charged: 136, 170, 186, 197, 247, 251, 261,

10

It is expected that changes in the charged amino acid residues will entail the greatest effect on the immunological potential of subtilisin 309.

15

#### Concluding remarks

SERIES A, the "data extraction" pages, TABLE III, list results from amino acid (AA) exchanges both ways i.e. there  
20 are sera towards both variants in TABLE III, and these have been tested with their immunogen and other variants comprising changes in the same position(s).

Looking at changes from WT to a variant the following effects  
25 are seen:

In the following the terms "essential", "critical", and "present" are used in connection with the amino acids in specified positions. These expressions have the meanings as  
30 defined in Geysen et al. Science 135 (1987)1184-90.

I. AA no. 120 is not "essential" in WT but becomes so in the variant.

35 AA no. 235 same as for 120 !  
AA no. 271 same as for 120 !



II. AA no. 251 is "essential" in WT but not in variant.

III. AA no. 181 is showing heteroclitic effect in change D181N and is "essential" in backwards change N181D.

5 IV. AA no. 136 is giving big impact on response both ways of exchange.

AA no. 170 same as for 136.

AA no. 195 same as for 136.

10 The following exchange data can be segmented in more or less two groups (of 13 and 11 respectively) :

V. Rows 9, 11, 12, 13, 14, 15, 17, 19, 20, 21, 26, 27 and 32 exhibits effects that would be expected from the calculated accumulated effects in single mutations.

VI. Rows 10, 16, 18, 22, 23, 24, 25, 28, 29, 30, and 31 exhibit effects that would be not expected from calculated accumulated effects in single mutations.

20

It is noted that V. and VI. have been calculated without including AA no. 36, as there are no two-way data on this change. Therefor rows 24, 25, 27, 29 and 32 may in subsequent calculations including AA no. 36 exchange come out differently.

All AA's with data both ways line up as participants in some epitope. Their impact on recognition and binding by antibodies are largely different, but none are without any effect.

Groups I. and II. illustrate how some AA's are non-essential, whereas other in the same positions are essential.

35 From this it seems as if the tested changes in AA's 120, 235 and 271 create essential AA's, maybe even epitopes in the variants.

Also, it seems as if change of no 251 removes an essential AA.

5 This may in humans lead to a reduced allergenic reaction to the new variant as compared to the reaction to the wild type enzyme. After production of new antibodies towards the variant molecule, there may still be a low reaction that anyhow should be restored with full strenght upon switch back to WT  
10 exposure (both by anti-WT and anti-variant antibodies).

The most interesting group is III. where change of no. 181 gives a heteroclitic effect (i.e. the anti-WT sera reacts stronger with the variants than with its own WT immunogen),  
15 and this AA seems to be essential to the anti-variant sera.

Therefore this seems to be a very important position, which upon change can create increased response, not only in individuals that are exposed to the molecule on a first-time-  
20 basis, but also individuals already having antibodies towards the WT enzyme can be expected to react even stronger with the variant.

This means that from an immunological view a change in this  
25 position should be avoided.

The group IV. shows changes providing antisera that both ways react strongest with their own immunogen. A change in both ways exhibits decreased response, and the responses are restored upon returning to their own immunogen.  
30

This may in humans mean an immediate lowering of response upon switch to variant, but as new anti-variant antibodies appear the response may be restored.

35

From an immunological viewpoint these changes seem to be neutral or even beneficial.

The remaining rows in Table III partly confirms the above, and partly illustrate that simple accumulation of effects cannot be expected in multiple AA exchange variants. Further analysis is needed to confirm any accumulation of immunological effects.

Using molecules wherein a single or a few amino acids have been changed the following effects were found:

- 10     1. In specific positions certain amino acids seems not to be essential to the epitope, whereas other may be.
2. In specific positions all tested amino acids seem to be essential to the epitope.
- 15     3. Exchange of one amino acid for another can give a heteroclitic effect. Furthermore the new amino acid may be essential to the "variant" molecule.
- 20 From these findings the following responses (incl symptoms) may be seen, if an individual already sensitive to the molecule of origin is exposed to the altered molecule(s) :
  - i     No change immediately, but shortly later an increased response. Upon switch to exposure to molecule of origin the response is restored.
  - 25     ii Lowering of the response upon change. Upon switch back to exposure to molecule of origin restoration of response.
  - 30     iii Increase in response upon change. Upon switch back to exposure to molecule of origin an immediate drop is seen, that finally resumes the original strenght of response before the change to the variant.
  - 35     iv Initially a drop in response, that is being restored.

Upon switch back to molecule of origin a drop in response that very soon is being restored.

From an immunological view the preferred switch will be of the group II type, but also a group IV type of change is acceptable.

Although the present invention has been illustrated in connection with certain specific embodiments, this is in no way to be construed that it should be limited to these embodiments, the invention being defined by the appended claims and the whole of the specification.

**PATENT CLAIMS**

1. A method of producing a protein variant evoking a lowered immunogenic response in animals including man in comparison to the response evoked by its parent protein,  
5 whereby said protein is epitope mapped using immunological and proteochemical methods, epitopes are determined, and at least one of said epitopes is changed through mutation of a DNA molecule coding for the expression of said parent protein or synthesis of a DNA molecule coding for the expression of  
10 said variant protein, said mutated or constructed DNA molecule subsequently being inserted into a vector for transformation of transfection into a suitable host, wherein said vector is functional or whereby said mutated or constructed DNA molecule is integrated functionally into the genome of  
15 said host, said protein variant is expressed in the host, and recovered.

2. The method of claim 1, wherein said protein is an industrial enzyme.

20

3. The method of claim 2, wherein said enzyme is a detergent enzyme, such as a protease, lipase, cellulase, amylase, or oxidase.

25 4. The method of claim 2, wherein said enzyme is a process enzyme, such as an amylase, lyase, lipase, or cellulase.

5. The method of claim 1, wherein said protein is a  
30 medicinal protein, such as a hormone, or medicinal enzyme.

6. A protein variant produced by the method of any of the claims 1 to 5.

35 7. The protein variant of claim 6, selected from the group comprising industrial enzymes, such as detergent enzymes, e.g. proteases, lipases, cellulases, amylases, or

8. The protein variant of claim 7, wherein said protein  
5 is a protease.

9. The protease variant of claim 8, wherein said protease is a subtilisin protease.

10 10. The protease variant of claim 9, representing a variant of a parent enzyme selected from subtilisin BPN', subtilisin amylosacchariticus, subtilisin 168, subtilisin mesentericopeptidase, subtilisin Carlsberg, subtilisin DY, subtilisin 309, subtilisin 147, thermitase, aqualysin, Bacillus PB92 protease, proteinase K, Protease TW7, and Protease TW3.

11. The protease variant of claim 10, wherein the parent enzyme is subtilisin 309.

12. The protease variant of claim 10, wherein the parent subtilisin is subtilisin 147.

13. The protease variant of claim 10, wherein the parent subtilisin is subtilisin Carlsberg.

14. The protease variant of claim 10, wherein the parent subtilisin is Bacillus PB92 protease.

30 15. A subtilisin protease variant, wherein the immunological potential has been changed in comparison to the parent protease, for example in that, in said protease changes have been performed among the amino acid residues at any one or more of positions

35 127, 128, 129, 130, 131, 136, 151, 152, 153, 154, 161, 162,  
163, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 186,  
193, 194, 195, 196, 197, 247, 251, 261,

by deletion, substitution, or insertion (single or multiple) adjacent to the indicated positions, whereby said subtilisin protease has an immunological potential lower than that of said parent protease.

5

16. The protease variant of any of the claims 8 to 15, characterised in that it possesses at least one mutation affecting an amino acid residue occupying a position chosen from the group of positions

10 127, 151, 152, 153, 154, 168, 169, 173, 174, 175, 176, 193, 196.

17. The protease as claimed in any preceding claim, further characterised in that it contains at least one or  
15 more sets of mutations affecting amino acid residues occupying a position chosen from the group of sets of positions:

36+209, 89+120, 136+170, 120+235, 170+195, 36+89, 89+235, 136+195, 181+222, 195+251, 209+222, 235+251.

20

18. The protein variant of claim 6, selected from the group comprising medicinal proteins, e.g. hormones, e.g. insulin, HCG, or growth hormone.

25 19. The protein variant of claim 6, selected from the group comprising medicinal enzymes, e.g. factor V, factor VII, factor VIII, or other proteins, e.g. interleukins, or interferons.

30 20. A composition comprising any protein variant according to any of claims 6 to 19.

21. The composition of claim 20, wherein said composition is a detergent compositions.

35

22. The composition of claim 20, wherein said composition is a compositions for use in preventive and/or

alleviating therapy and/or diagnosis of various conditions 'in' the animal body, including man.

23. Use of a protein or composition according to any of  
5 the claims 6 to 22 in detergents or for preventive and/or  
alleviating therapy and/or diagnosis of various conditions in  
the animal body, including man.



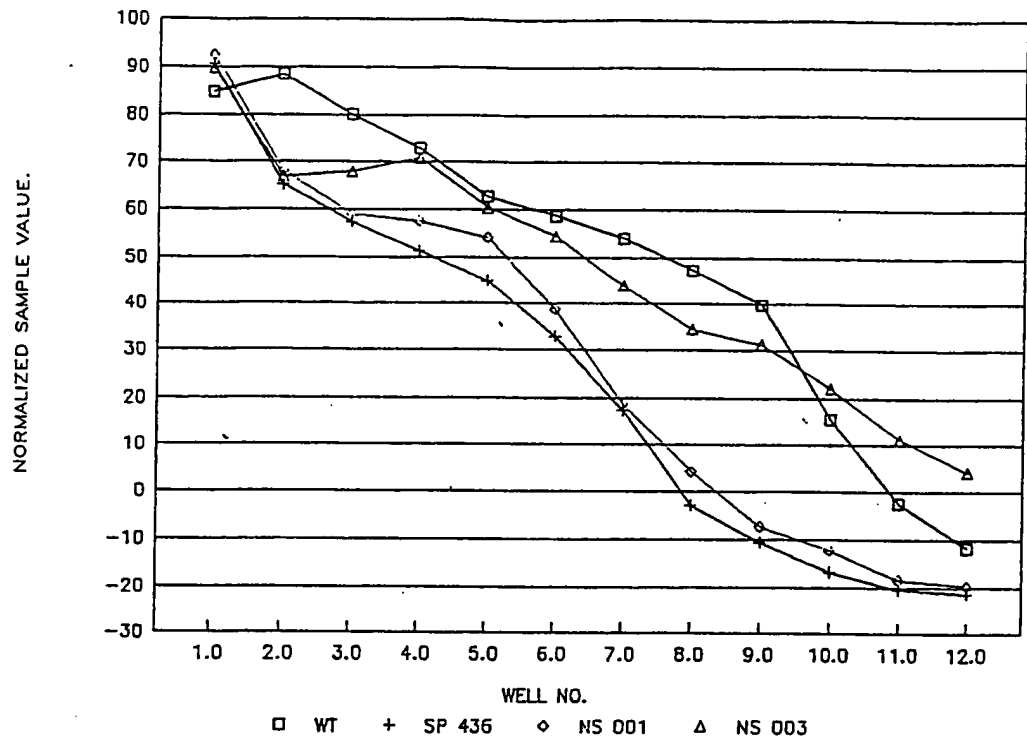


FIG. 1

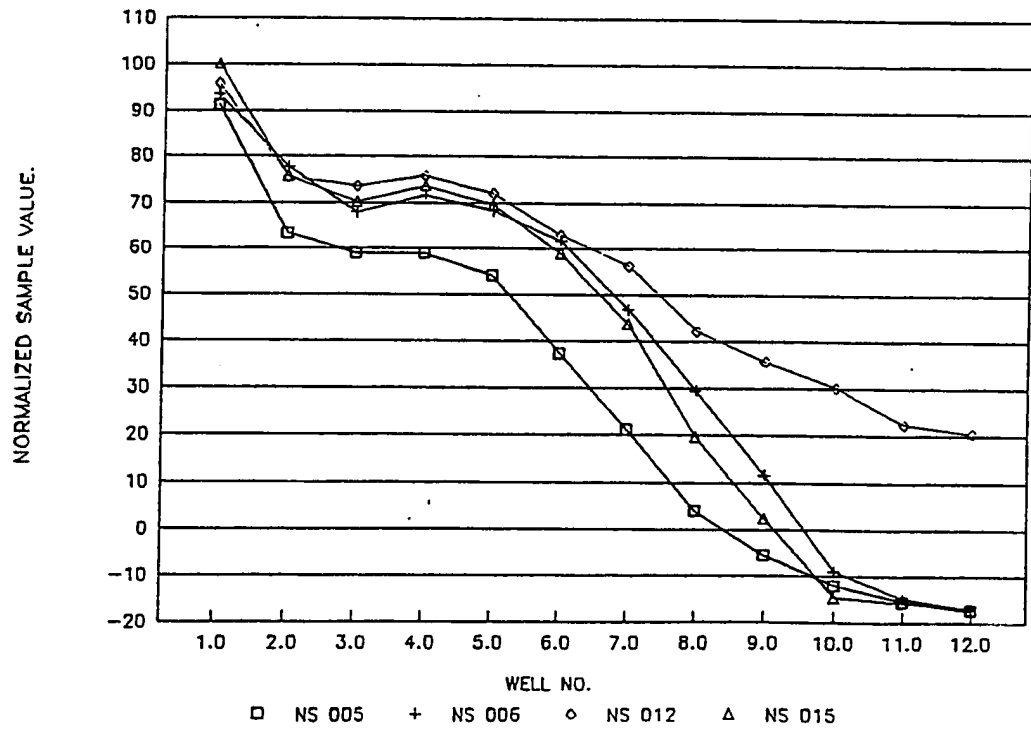


FIG. 2

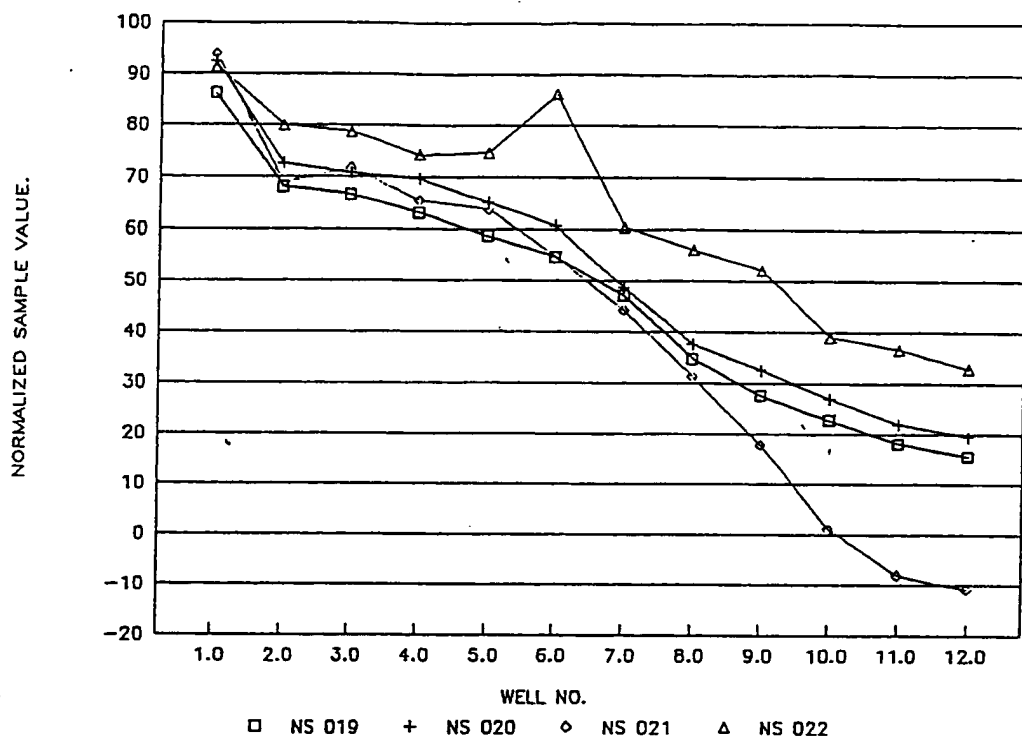


FIG. 3

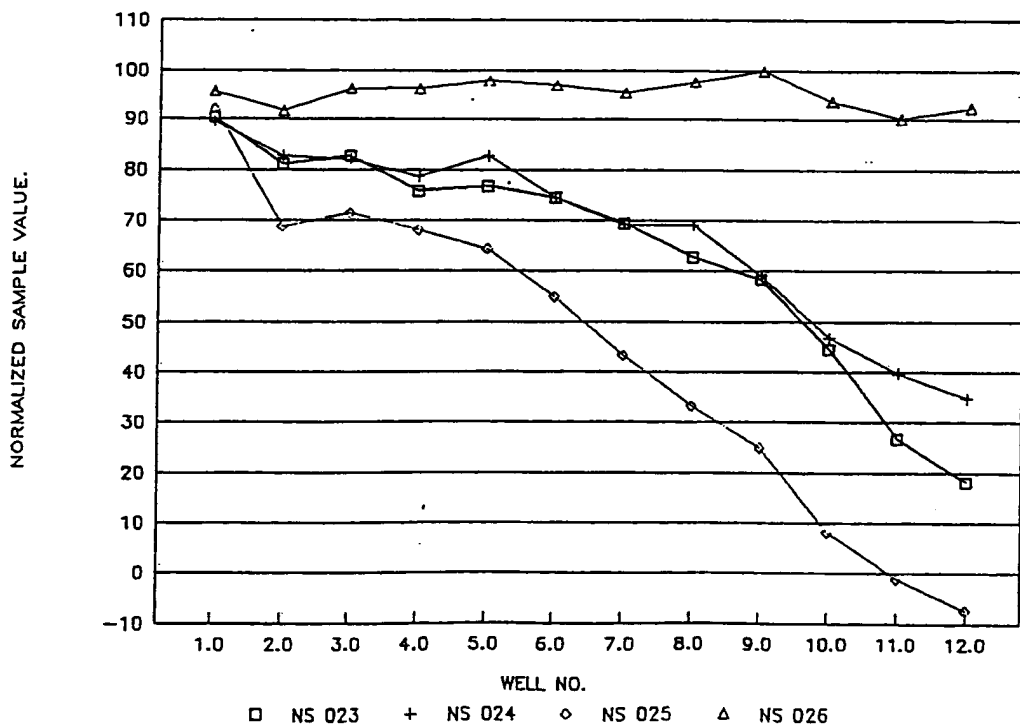


FIG. 4

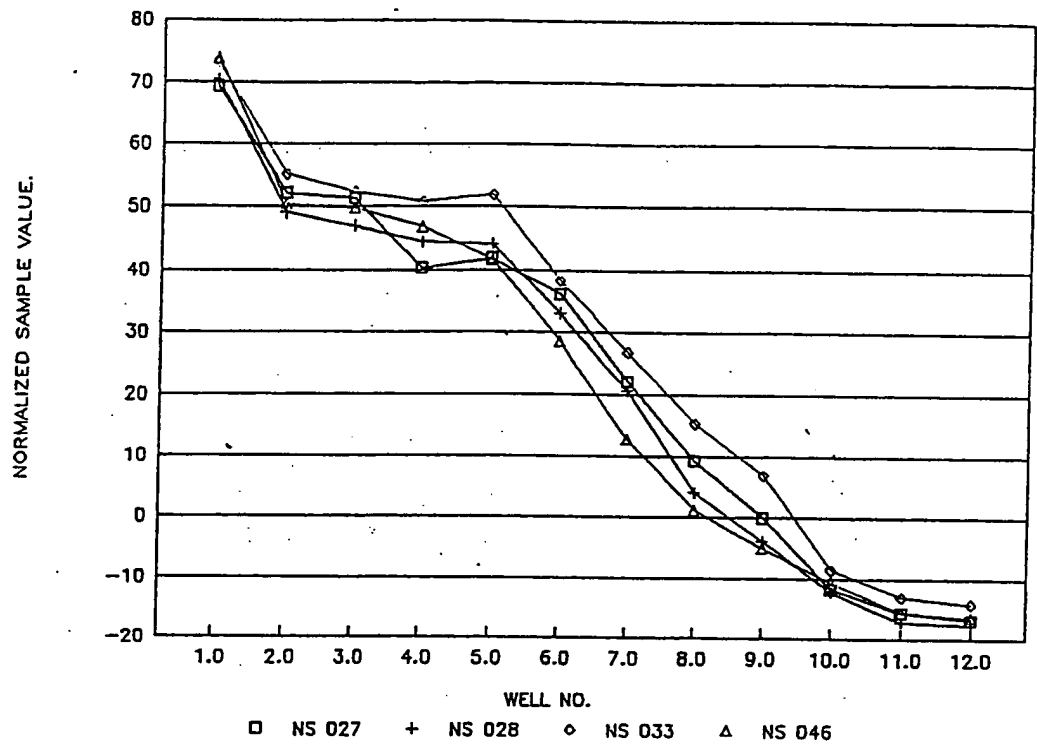


FIG. 5

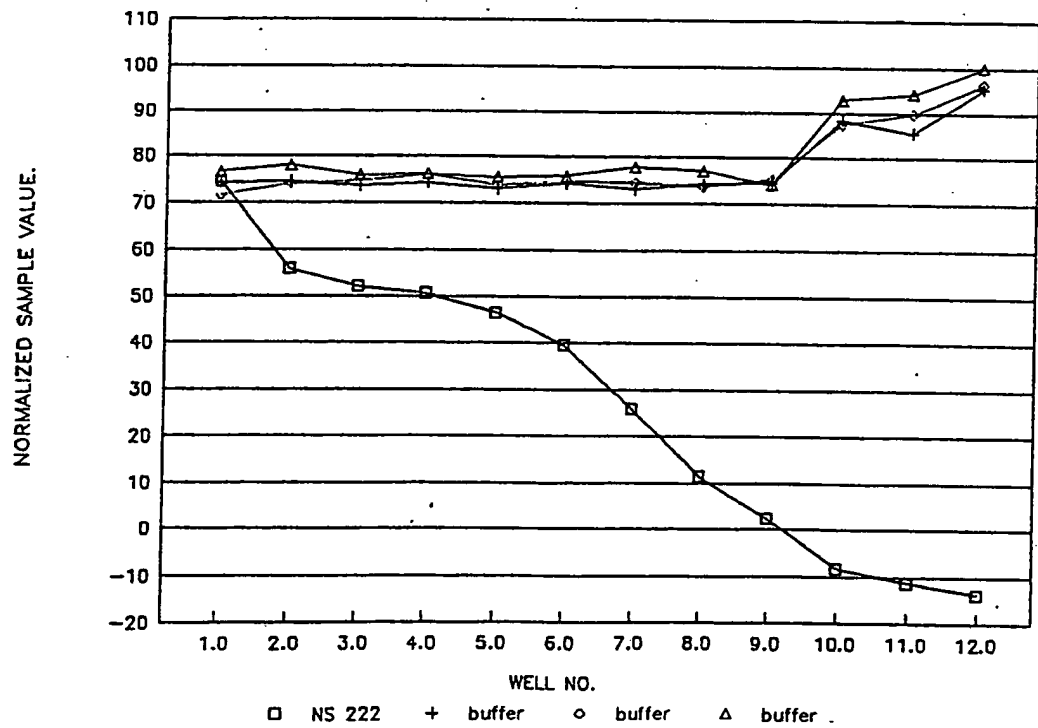


FIG. 6

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC5: G 01 N 33/53, C 07 K 3/00, C 12 N 9/00, 9/56, 15/12		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC5	G 01 N; C 07 K; C 12 N	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in Fields Searched <sup>8</sup>		
SE,DK,FI,NO classes as above		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	US, A, 3903068 (MICHAEL A. RUTTENBERG) 2 September 1975, see especially column 2, lines 15-32 --	1-9, 18-23
Y	Dialog Information Services, File 73, Embase, Dialog accession no. 7753695, Embase accession no. 90182676 Arlan L.G. et al: "Antigenic and allergenic characterization of the enzymes alcalase and savinase by crossed immunoelectrophoresis and crossed radioimmunoelectrophoresis", & Int. Arch. Allergy Appl. Immunol. (Switzerland), 1990, 91/3 (278-284) --	1-9, 18-23
Y	Dialog Information Services, File 154, Medline 85-92 Dialog accession no. 07039431, Medline accession no. 89341431, Walsh BJ et al: "A method for the detection of IgE binding sequences of allergens based on a modification of epitope mapping", & J Immunol Methods Jul 26 1989, 121 (2) p275-80 --	1-9, 18-23
<p>* Special categories of cited documents:<sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
27th March 1992	1992 -03- 3 0	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	Mikael Bergstrand	

Form PCT/ISA/210 (second sheet) (January 1985)

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Dialog Information System, File 154, Medline 85-92, Dialog accession no. 06879622, Medline accession no. 89181622, Favre C et al: "Epitope mapping of recombinant human gamma interferon using monoclonal antibodies", & Mol Immunol Jan 1989, 26 (1) p17-25 --	1-9, 18-23
A	Dialog Information System, File 73, Embase, Dialog accession no. 5035050, Embase accession no. 82036005, Zachariae H et al: "Detergent enzymes and occupational safety. Observations on sensitization during Esperase (Reg. trademark) production", & Allergy (DENMARK), 1981, 36/7 (513-516) -- -----	1-23

Form PCT/ISA/210 (extra sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00382

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the Swedish Patent Office EDP file on 28/02/92  
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 3903068	75-09-02	NONE	